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Congenital Neutropenia: *In Vitro* Growth of Colonies Mimicking the Disease

(hematopoietic/defective stem cell/abnormal clone/differentiation)

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ABSTRACT **Congenital neutropenia is a lethal disease characterized by recurrent infections beginning in the neonatal period, absence of neutrophils in the peripheral blood, eosinophilia, and monocytosis. The bone marrow shows an apparent "maturation arrest" of the neutrophil series at the promyelocyte stage. Granulocytic colonies grown *in vitro* in soft agar medium show normal development of eosinophilic colonies and monocyte–macrophage colonies, but defective neutrophil maturation. The abnormal colonies observed contained only myeloblasts and promyelocytes. Thus, it seems to have been possible to mimic *in vitro* the abnormal differentiation that is observed *in vivo*.**

Blood and bone-marrow cells of animals and man (1–4) can give rise to granulocytic colonies when grown *in vitro* in soft agar medium with appropriate stimuli. Colonies can be grown from blood and marrow samples obtained from patients with hematologic and nonhematologic diseases, as well as from normal persons (2–7). The colonies arising from normal human blood and marrow are comprised of eosinophils, neutrophils, and monocyte–macrophages. We had the opportunity to study the marrow cells from a patient with congenital neutropenia using the soft agar technique. The marrow from this patient exhibited an abnormality of differentiation in the soft agar culture that seems precisely to mimic the abnormality of differentiation apparent from study of smears of the bone marrow itself. To our knowledge this represents the first observations *in vitro* that seem to reproduce the morphological features of an apparent inborn error of metabolism reflected as an abnormality of differentiation of a cell line from the bone marrow.

Congenital neutropenia is a disorder characterized by recurring infections, beginning soon after birth, and by absence of neutrophils in the peripheral blood. Eosinophilia and monocytosis are regularly observed, especially during acute bacterial infections. The bone marrow of these children shows an apparent "maturation arrest" at the myeloblast–promyelocyte level. Only very rarely are neutrophils to be seen beyond this stage in either marrow or blood. Death usually occurs before the age of 2 years. This is an extremely rare disease with only 20 cases having been reported, since Kostman's original study (8) probably included such cases in 1956.

MATERIALS AND METHODS

In this investigation, marrows from one patient with congenital neutropenia and two children with nonhematologic disorders were compared. After appropriate preparation with local procaine anesthesia, 1 ml of marrow was aspirated from the tibia or iliac crest into a syringe previously rinsed with beef-lung heparin (1:1000, Upjohn). The bone marrow specimen was centrifuged at 1000 rpm for 5 min (183 × *g*). After centrifugation, the bone-marrow cells were removed with a Pasteur pipette and washed three times in McCoy's 5A medium. The culture method described by Pike and Robinson (2) was used. The medium used to prepare underlayers and overlayers is McCoy's 5A tissue culture medium (Microbiological Ass., Bethesda, Md.) to which has been added 15% fetal-calf serum and supplements of amino acids and vitamins.

Colony growth was stimulated by plating 1 ml of McCoy's 5A medium mixed in a 9:1 concentration with boiled 5% agar (Difco-Bacto-agar) to give a final concentration of 0.5%. Blood leukocytes were obtained by venipuncture and allowed to sediment at room temperature. After sedimentation, the cell-rich plasma was removed. The number of nucleated leukocytes varied from 1.0×10^7/ml to 3.0×10^7/ml. These human peripheral cells with the plasma were added to the mixture to achieve a final concentration of 1.0×10^6/ml. This preparation constituted the feeder layer, which was incubated in plastic petri dishes (35 by 10 mm, Falcon Plastics, Los Angeles, Calif.) at 37.5° (constant flow of 10% CO_2). The washed bone-marrow cells were added to the mixture described above, except that the agar concentration was 0.3% instead of 0.5%. Then, 2×10^5 of the marrow cells per ml were plated on top of the previously prepared leukocyte underlayers and incubated for 3 weeks. Colony counts were performed repeatedly with an inverted microscope at ×25 magnification. Counts were made on days 17, 19, and 21 after plating. With normal bone-marrow cells, only colonies of 50 cells or more are counted, but with bone-marrow cells from the patient, because of abnormalities to be described below, colonies of 40 or more cells sometimes had to be accepted. By use of a Zeiss camera attached to the inverted microscope, a photographic study and record of these colonies in the soft agar gel was made at ×25 and ×160 magnification. The photographed colonies were then gingerly removed, along with a small amount of soft agar, with a microhematocrit tube. The colonies thus extracted were squashed and smeared between glass coverslips. After air drying, the preparations were stained with Romanowsky stains, histochemical studies were made with peroxidase, or periodic acid–Schiff reactions were performed.

RESULTS

Colonies grew from the bone marrow of the patient, as well as from the marrow of the two control children. Underlayers

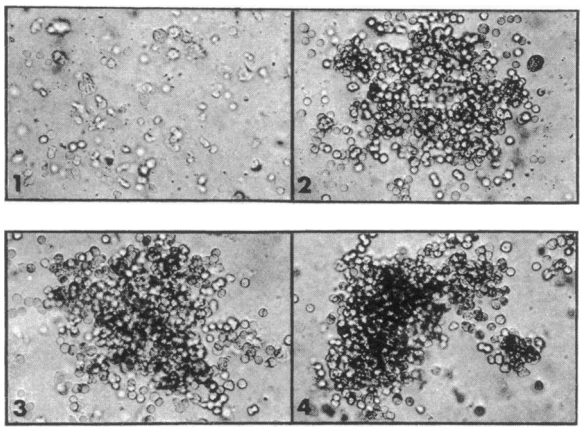

FIG. 1. Neutrophil and eosinophil colonies in soft agar. Colonies: *1:* abnormal neutrophil precursors; *2:* control neutrophils; *3:* patient's eosinophils; *4:* control eosinophils.

were prepared from the same volunteer whose cells had proved regularly to support colony growth from marrow cells from normal controls. The number of colonies obtained from the marrows of the two control children and from the patient ranged from 26 to 94 per 2×10^5 marrow cells plated. These values for both the normal controls and the patient studied here fell within the range expected for normal and nonleukemic marrow in our laboratory, and they further agree well with the number of colonies obtained by others from similar preparations. The number of colonies from the patient's marrow tended to range on the low side of the normal limits. Colonies usually contained about 300–1200 cells. The colonies of the two control children were exclusively of the so-called tight variety (2). From the normal bone-marrow preparations, colonies of eosinophils, neutrophils, and mononuclear cells were readily identified. In this system monocytic colonies appeared largely as macrophages; even though the monocyte count of the patient was high, the colonies did not usually appear to contain a majority of monocytes, but rather were predominantly macrophages. Certain colonies from the congenital neutropenic patient contained 40–75 cells (Table 1). These colonies were of the loose type (Fig. 1), and the number of this type of colony was low. Of 100 colonies studied from the growth of the patient's marrow, about 5% were of this loose type; they apparently contained neutrophilic precursors. Microscopic examination of the stained smears from these colonies showed clear evidence of promyelocytes, but an absence of neutrophilic differentiation beyond the promyelocyte stage. Since the patient's marrow also contained small numbers of promyelocytes—about 3%—but no myelocytes or neutrophils, it would seem that in this instance findings from study of bone marrow *in vivo* of the disease from which the patient suffers have been reproduced *in vitro*, and that a characteristic abnormality may be reflected in some colonies as an arrest of differentiation at the promyelocyte stage.

DISCUSSION

The results of our study indicate that cells from patients with congenital neutropenia can be grown *in vitro* by the soft agar gel technique. The finding that colonies obtained from the patient's marrow are almost exclusively of the eosinophilic and mononuclear type suggests that the defect in the neutrophil precursor cells is revealed in the *in vitro* study of cell differentiation. The neutrophilic elements are unable to proliferate and mature with the same efficiency as do cells in preparations made from hematologically normal children. The abnormality of the neutrophil precursors is probably reflected both in the small number of neutrophil colonies and in the nature of the loose colonies in which cells mature only to promyelocytes. These loose colonies in the culture of the patient's marrow regularly contained only 40–75 cells at a time when in cultures of the normal marrow the neutrophil colonies usually contained 300 or more cells.

The fact that these abnormal colonies are comprised entirely of myeloblasts and promyelocytes, and that neutrophil differentiation was not seen while many colonies showing full eosinophil differentiation seemed to develop normally in the cultures, indicates not only that neutrophil differentiation *per se* is deficient in this patient, but that among cells with the myeloblast morphology are probably cells already committed to propagate only one specific line of myeloid elements. The eosinophil colonies contained cells that morphologically would be called early promyelocytes, but proliferation and maturation to eosinophils seems to proceed normally. The best evidence indicates that the colonies observed in soft agar preparations represent a clonal development, together with differentiation from a single cell (9). With this in mind, one can, from the studies reported here, draw conclusions relevant to the stage of differentiation at which the cellular abnormality in this patient with congenital neutropenia exerts its influence. Inferences from these findings also have

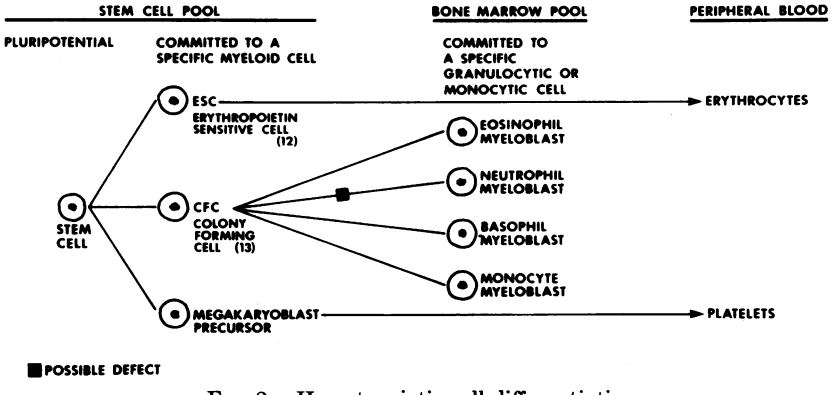

FIG. 2. Hematopoietic cell differentiation.

TABLE I

Number of Cells in Different Types of Colonies

		Fetal-calf 10% serum		Normal 10% serum		Patient 10% serum	
		No. colonies per plate	No. cells per colony	No. colonies per plate	No. cells per colony	No. colonies per plate	No. cells per colony
Patient	N	38±6*	40-75	34±8	40-75	38±5	40-75
	M+E		>300		>300		>300
Control	N	85±5	>300	83±6	>300	79±9	>300
	M+E		>300		>300		>300
Control	N	74±6	>300	73±8	>300	66±9	>300
	M+E		>300		>300		>300

*Quintuplicate plates for each experiment
N---→ Neutrophils
E---→ Eosinophils
M---→ Monocytes

significance in understanding the normal differentiation of neutrophils, and the abundance of eosinophils and monocytes so characteristic in blood and bone marrow of these patients. Presented on Fig. 2 is a scheme of differentiation that seems compatible with both the clinical and hematologic findings and with the results of our cultures of marrow in soft agar. Since eosinophilic and mononuclear cell development proceeds normally in the patient, a pluripotent stem cell and the entire lineage of eosinophils and mononuclear cells are presumably normal and respond normally to exogenous influences like those present in the soft agar environment. The arrested development involves only the neutrophil line, and is reflected in the small number of neutrophil colonies and in the abnormal morphology of those colonies that do become established. This finding indicates that these patients have a deficit in one of the proliferative cells, the committed stem cell capable of differentiating only to neutrophils. Production of neutrophils from marrow cells in normal persons from such a precursor accounts for many, if not most, colonies developing to neutrophils directly in soft agar. In a culture of the patient's marrow, some such colonies are seen but are abnormal in appearance and reveal the arrested development that also is seen in the bone marrow (Table 1, Fig. 1). We might call such a cell a neutrophilic myeloblast or committed myeloblast. In parallel with terminology for other hematopoietic elements, this cell can be presumed to be committed to the neutrophil line, as opposed to either eosinophil or monocyte lines, among the colony-forming cells. A few of the abnormal neutrophil precursors do become established and are seen as abnormal colonies in soft agar, as illustrated in Fig. 1. The colony-forming cell that contains the abnormality does not become established regularly or does not develop efficiently to the neutrophil colony. This abnormality results in a relative increase in eosinophil- and monocytic–macrophage-containing colonies. When normal marrow is cultured, many neutrophil colonies derived from the colony-forming cell and neutrophil-containing colonies predominate. Many of the neutrophilic colonies from normal marrow culture probably derive directly from this committed neutrophil myeloblast.

Mixture of the patient's serum with normal bone-marrow cells did not affect colony growth of the normal bone marrows (Table 1), nor did mixture of normal serum with the patient's marrow cells correct the abnormal development in soft agar. We did not attempt to determine whether the patient's cells would function normally as a feeder layer, but this would be a most important experiment, which should be done at the earliest opportunity. Barak *et al.* (10) studied by similar techniques a patient with genetically determined infantile agranulocytosis, culturing the marrow cells with spleen supernatant. In this system, the patient's marrow could differentiate only to neutrophils. Their patient, however, was quite different from the one studied here, since his marrow could produce sufficient numbers of neutrophils and neutrophilic precursors *in vivo* to give a neutrophilic infiltration in Rebuck skin window. Our patient could not produce such cells and Rebuck skin window, as well as blood and bone marrow, contained no neutrophils. In the patient of Barak *et al.*, for example, the nitroblue tetrazolium test was positive among the neutrophils during infection, whereas during infection our patient showed only nitroblue tetrazolium-positive monocytes (11).

It seems likely that if pluripotent stem cells, colony-forming cells, or even committed neutrophil myeloblasts could be replaced in a patient such as ours, and if the allograft rejection of these cells can be obviated, the disease might be corrected by a form of cellular engineering.

We conclude that our findings have permitted the first characterization of a congenital, presumably genetically determined, defect by use of the soft agar culture technique. These findings also represent the first demonstration of a deficiency of colony growth and a presumed proliferative defect to be reported in a hematologic disease other than leukemia.

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